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Bioprocess Considerations for Production of Secondary Metabolites by Plant Cell Suspension Cultures

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Abstract Plant cell culture provides a viable alternative over whole plant cultivation for the production of secondary metabolites. In order to successfully cultivate the plant cells at large scale, several engineering parameters such as, cell aggregation, mixing, aeration, and shear sensitivity are taken into account for selection of a suitable bioreactor. The media ingredients, their concentrations and the environmental factors are optimized for maximal synthesis of a desired metabolite. Increased productivity in a bioreactor can be achieved by selection of a proper cultivation strategy (batch, fed-batch, two-stage *etc.*), feeding of metabolic precursors and extraction of intracellular metabolites. Proper understanding and rigorous analysis of these parameters would pave the way towards the successful commercialization of plant cell bioprocesses.

Keywords: bioprocessing, bioreactors, phytochemicals, plant cell cultures, secondary metabolites

INTRODUCTION

Higher plants are inexhaustible sources of a wide range of biochemicals such as flavors, fragrances, natural pigments, pesticides and pharmaceuticals. This seemingly unrelated class of chemicals could be grouped together under a broad heading of plant secondary metabolites. Currently many of these compounds are isolated by solvent extraction from the naturally grown whole plants. The continued destruction of plants has posed a major threat to the plant species getting extinct over the years. Clearly, the development of alternative and complimentary methods to whole plant extraction for the production of these compounds, especially of medicinal value, is an issue of considerable socio-economic importance. These factors have generated considerable interest in the use of plant cell culture technologies for the production of pharmaceuticals [1]. Since about last two decades, plant cell and tissue culture technology has made it possible to gradually replace the whole plant cultivation as a source of useful secondary metabolites. In plant cell culture, the isolated cells from the whole plant (or parts derived thereof) are cultivated under appropriate physiological conditions and the desired product is extracted from the cultured cells. The recent developments in plant tissue culture techniques and their processing have shown promising results to improve the productivity by many folds.

The production of pharmaceuticals by plant tissue culture offers a number of advantages such as:

- Control of supply of product independent of the availability of plant itself.
- Cultivation under controlled and optimized conditions.
- Strain improvements with programs analogous to those used for microbial systems.
- No need of the use of harmful herbicides and pesticides.
- Possibility of synthesizing novel compounds, not present in nature, by feeding of compounds analogous to natural substrates.
- No dependence on climate, and geographical location etc.

CELL SUSPENSION CULTURES

The first step in plant tissue culture is to develop a callus culture from the whole plant. Callus is a proliferated mass of cells without any significant differentiation. A callus can be obtained from any portion of the whole plant containing dividing cells. To maximize the formation of a particular compound, it is desirable to initiate the callus from the plant part that is known to be a high producer. The callus tissues can be extracted by suitable solvents to isolate the desired compound. However, from an engineering perspective, cell suspension cultures have more immediate potential for industrial application than plant tissue and organ cultures, due to extensive expertise which has been amassed for submerged microbial cultures. While tissue and root cultures offer genetic stability as well as, in some instances, superior metabolic performances over suspension cultures of the cell lines, the development of appropriate bioreactors and operating techniques for these

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systems involve high investment and laborious experimentation [2]. Accordingly, most of the research efforts have been directed towards commercialization of plant cell suspension cultures. Often, suspension and callus cultures produce a desired compound initially, but after a few passages the capacity to synthesize the product decreases considerably. This loss of productivity may be due to either inadequate nutrition or genetic variations. Therefore, it is necessary to select and preserve the high-yielding cell line from the mixed population of the plant cell cultures.

A suspension culture is developed by transferring the relatively friable portion of a callus into liquid medium and is maintained under suitable conditions of aeration, agitation, light, temperature and other physical parameters. However, various strategies may have to be adopted to obtain a fairly homogeneous suspension culture. Initiation of suspension cultures of *Podophyllum hexandrum* has, for example, been found to be difficult in the authors' laboratory due to clumping of cells, browning of culture medium and drop in medium pH. These were addressed by inclusion of pectinase, which reduced the clumping of cells and polyvinylpyrrolidone, which stopped browning of the culture medium [3]. It was observed that the development of an active and segregated population of plant cells in suspension cultures was dependent on the zone of collection of the plant species, the genetic make-up, the callus texture and the medium/hormone combinations.

Mitsui Petrochemical Industry, Japan was the first to produce shikonin (a dyestuff) on commercial scale. The company also succeeded in using plant cell culture technology to produce berberine. Recently, a very significant milestone in culturing plant cells in large bioreactors (32 kL capacity) was achieved by Samyang Genex Corporation, Republic of Korea for commercial production of 20 kg paclitaxel per year under the trade name of Genexol. While the large-scale cultivation of plant cell suspension cultures is desirable for industrial production of plant-derived biochemicals, the production technology comparable to that used for microbial systems needs to be further developed. Although the basic equipment- and process- related requirements for suspension cultures of plant cells are similar to those of submerged microbial cultures, some of the features used for microbial cultures are not suitable for plant cell cultures because of striking differences in the nature and growth pattern of the two types of cells (Table 1). The implications of these differences on culturing of the plant cells are summarized in Table 2 [4]. Certain biological and engineering considerations are normally addressed before embarking on the mass scale propagation of plant cells for commercialization.

Plant cells in suspension cultures often undergo spontaneous genetic variation in terms of accumulation of secondary metabolites, which leads to heterogeneous population of cells in a suspension culture. This variation, known as somaclonal variation, has posed a commercial hurdle to the large scale production of secondary metabolites. The genetic basis of somaclonal variation has not been extensively studied; however, it has been observed to be advantageous in crop improvement. Establishment of a high yielding genetically stable cell line would provide a suitable means for the large scale production of plant metabolites. The regulatory mechanisms of secondary metabolism have also been poorly understood. Yields of metabolites will improve with proper understanding of regulatory mechanisms, plant cell differentiation, intracellular organization, and cell physiological characteristics, as these are linked to secondary metabolism. Increasing awareness in metabolic engineering of various metabolic routes would eventually lead to improvement of product accumulation by the cultured cells [5].

ENGINEERING CONSIDERATIONS

Plant cell suspensions can now be successfully cultivated at small scale in bioreactors of various configurations. However, many of the unique properties of plant cells in culture such as sensitivity to shear, slow growth rates, and low oxygen requirements are manifested in complex ways at large scale cultivation. As the scale of operation increases, mixing inside the bioreactor vessel becomes difficult, resulting in non-uniform concentration of the nutrients and limited oxygen transfer to res-

Table 1. Comparison of the characteristics of microbial (yeast) and plant cells

Characteristics	Typical microbial (yeast) cell	Typical plant cell
Shape	Spherical, cylindrical	Spherical, cylindrical
Size (μm)	2-10	50-100
Growth pattern	Single cells	Mostly aggregates
Doubling time (h)	1-2	20-100
Oxygen requirement (vvm)	1-2	< 0.5
Fermentation time	2-10 days	2-4 weeks
Water content (%)	80	> 90
Shear sensitivity	*Insensitive*	Sensitive
Regulatory mechanisms	Complex, mostly known	Highly complex, mostly unknown
Genetic makeup	Stable	May be highly variable
Product accumulation	Often extracellular	Mostly intracellular

Table 2. Differences between the characteristic features of plant and microbial cells and their implications for bioreactor design

Characteristic features of a typical plant cell	Implications for reactor design
Lower respiration rate	Lower oxygen transfer rates required
More shear sensitive	May require operation under low-shear conditions by, for example, employing low-shear impellers and bubble-free aeration
Growth as aggregates	May have mass transfer limitations that limit the availability of nutrients to cells within the aggregates
Aggregation important for secondary metabolism	An optimal aggregate size may be required for product synthesis by manipulation of media constituents and environmental conditions
Volatile compounds (*e.g.* CO_2 or ethylene) may be important for cell metabolism	May need to sparge gas mixtures containing them
Product synthesis may be non-growth-associated	May require a two-step cultivation system for maximal product synthesis

Adapted from [4].

piring cells. Changes in the rheological nature of the fluid, wall growth, and clumping of cells resulting in sedimentation, lead to suboptimal utilization of the bioreactor. These problems necessitate a more rigorous analysis of bioreactors to be used for the large scale cultivation of plant cells for metabolite production. A number of reviews have dealt with these unique problems associated with plant cell cultivation in bioreactors [6-12].

Aggregation

Plant cells are significantly larger and slower growing cells than most microbial organisms. Due to larger size of a plant cell, it is capable of withstanding tensile strain but is sensitive to shear stress. Aggregation is common, largely due to failure of the cells to separate after division, although the secretion of extracellular polysaccharides, particularly in the later stages of growth, may contribute to increased adhesion [10]. This tendency of the plant cells to grow in clumps results in sedimentation, insufficient mixing and diffusion-limited biochemical reaction. This so-called cell-cell contact is desirable for the biosynthesis of many secondary metabolites by the plant cells in suspension cultures. Hence controlled aggregation of plant cells is of interest from process engineering point of view.

Mixing

Mixing promotes better growth by enhancing the transfer of nutrients from liquid and gaseous phases to cells and the dispersion of air bubbles for effective oxygenation. Although plant cells have higher tensile strength in comparison to microbial cell, their shear sensitivity to hydrodynamic stresses restricts the use of high agitation for efficient mixing. The high shear rate and shear time that accompany good mixing reduce the mean aggregate size, but also have an adverse effect on cell viability. Plant cells are, therefore, often grown in stirred tank bioreactors at very low agitation speeds.

Mixing of plant cells grown on a large scale is also hampered by the rheological characteristics of the culture broth [8]. Plant cell suspensions are viscous at high concentrations and behave like non-Newtonian fluids. Non-Newtonian rheological characteristics have been observed in cell suspensions of *Morinda citrifolia* [11], *Catharanthus roseus* [12], *Nicotiana tabacum* [13] and *Cudrania tricuspidata* [13]. Non-Newtonian behavior of the culture broth also restricts effective mass and heat transfer inside the bioreactor, leading to non-uniform nutrient concentration and temperature, and the development of dead zones inside the culture vessel. Excretion of polysaccharides at the later stages of cell growth, the extent and nature of which depend on the nature of the plant cells and the carbohydrate source used for growth, also results in a rapid increase in viscosity. Tanaka [12] investigated the effect of high oxygen supply in high cell density cultivation of *Cudriana tricuspidata*, a highly shear-sensitive cell line, and observed that the cell aggregates larger than 2 mm were damaged which impeded the cell growth. Mass transfer coefficient (K) for a model solid-liquid system (β-naphthol particles and water) was proposed as an intensity index of hydrodynamic stress effects on plant cells in suspension culture in place of a system consisting of plant cells and liquid medium. It was observed that normal cell growth took place at culture conditions with K values of less than 4.4×10^{-3} cm/sec. In the same study, the intensity of the culture broth mixing and air-bubble dispersion was measured by k_La in the presence of plant cells [12]. It was found that k_La and K were linearly correlated for the conditions used in all bioreactors; k_La was proportional to the αth power of K, where α varied with the type of bioreactor. The study concluded that a jar fermentor with a modified paddle type impeller without baffles was the most suitable device for culturing plant cells at high density. Inadequate mixing may lead to clumping of cells, thereby complicating the nature of the reacting system; also the inner cells of the clumps become nutrient deficient, which may have either an adverse or a positive effect on the cell growth and prod-

uct formation [6]. Adequate mixing can be achieved by proper design of the impeller; helical-ribbon impeller has been reported to enhance mixing at the high density of plant cell suspension cultures [14]. A low-shear impeller such as Setric impeller has been found to be effective for cultivation of shear-sensitive *Podophyllum hexandrum* in stirred tank bioreactor for production of podophyllotoxin [15].

Oxygen and Aeration Effects

Oxygen requirements of plant cells are comparatively lower than that of microbial cells due to their low growth rates. In some cases, high oxygen concentration is even toxic to the cells' metabolic activities and may strip nutrients such as carbon dioxide from the culture broth. Carbon dioxide is often considered an essential nutrient in the culture of plant cells and has a positive effect on cell growth. Hence, the factors that influence effective oxygen transfer in plant cell cultures must be carefully analyzed when a bioreactor system is being selected. The intensity of culture broth mixing, the degree of air bubble dispersion, the culture medium's capacity for oxygen, and the hydrodynamic stress inside the culture vessel affect proper aeration of the culture. Effects of aeration on plant cell suspension cultures have focused largely on the influence of k_La, the mass transfer coefficient, in which the aeration and agitation are linked. The k_La value gives a direct measure of effective oxygenation of culture fluid and helps one choose a suitable bioreactor system to culture plant cells. Increased viscosity of the culture broth decreases k_La and signals the need for intensive agitation of the culture for better mixing and oxygen transfer. A balanced analysis of mixing and oxygen transfer as reflected in k_La value is, therefore, required to achieve reasonable cell yield and product formation. Several inves-tigations have been made on the effect of mass transfer coefficients on the growth and product formation in plant cell suspension cultures in bioreactors [12,16]. The effect of initial k_La on growth and alkaloid production by suspension cultures of *Catharanthus roseus* was studied in 12.5 L stirred tank bioreactor using either a cross sparger or a sinter sparger, and a 6-bladed Rushton impellor for agitation [16]. It has been observed that, at higher k_La values, serpentine was produced when the cells were in the log phase, whereas production of serpentine and ajmalicine was maximum at k_La values of 16 h^{-1} and 4.5 h^{-1}, respectively [16]. The values of k_La, as mentioned earlier, has also been used for the selection of suitable bioreactor for *Cudrania tricuspidata* cell cultures [12].

High aeration may lead to severe foaming, which has considerable influence on the cell growth and secondary metabolite production [17]. Foaming of plant cell suspensions has been correlated with aeration rates and extracellular protein concentrations [18]. A number of antifoams such as, polypropylene glycol 1025 and 2025, Pluronic PE 6100, and Antifoam-C have often been employed to control foaming; however, in some cases this resulted in reduction in cell growth and product formation [18].

Shear Sensitivity

The perceived sensitivity of plant cells to hydrodynamic stress associated with aeration and agitation can be attributed to the physical characteristics of the suspended cells, viz. their size, the presence of thick cellulose based cell wall, and existence of large vacuoles. Mechanically agitated vessels may over-aerate plant cultures, in addition to damaging and breaking the cells through the hydrodynamic stress generated by aeration, agitation, shaking, pumping, and other operations. Low agitation and high aeration have been used in stirred tank bioreactors to supply oxygen in a reasonable mixing range [19]. The air-lift bioreactor has also been used to achieve better oxygen transfer and good growth. Bubble-free aeration of the culture fluid through a moving membrane provided another suitable alternative for transferring gas without inducing cell damage through shear stress.

The immediate consequence of the shear effect on plant cells is cell damage, which has been quantitatively measured by using a number of system responses such as reduction in cell viability [20], release of intracellular compounds [21], changes in morphology and/or aggregate patterns [22], and changes in metabolism [23]. The effects of hydrodynamic and interfacial stress on plant cell suspension cultures with various modes of quantitative analysis of system response at shake flask as well as bioreactor levels have recently been reviewed by Kieran and co-workers [24]. Although the studies conducted in this regard are highly system specific due to the variety of bioreactors employed, it is notable that the analysis of shear effects in a scaled-down version offers the greatest potential for successful scale-up of the results. The hydrodynamic environment is generally regulated by changing the rate or mode of aeration and/or agitation. The problem could also be solved by directing research effort to the development of shear-resistant cell lines [9].

OPTIMIZATION OF PROCESS PARAMETERS

Appropriate nutrients, their concentrations and environmental factors are known to enhance the yield and productivity of metabolites in plant cell suspension cultures. However, it is essential to study and quantify the effect of selected key medium components on growth as well as product accumulation and strike a balance between the two to enhance the yield and productivity. This is particularly important for plant secondary metabolites as conditions suitable for growth may adversely affect the product formation and vice versa.

Several methodologies can be applied for optimization of media components for the production of secondary metabolites. The first step in any bioprocess media optimization is the identification of relatively significant media components such as sugars, nitrogen compounds, minerals and growth factors as well as culture conditions and then to determine their optimum levels.

The classical approach of media design involves changing one component while maintaining the others at a constant level. This procedure is simple but time consuming, particularly for a large number of components, and it does not generally guarantee that the optimum obtained is the true optimum. Another drawback of this methodology is its inability to detect the interactions which normally exist among the media ingredients. A more rational approach is based on statistical techniques which can overcome the difficulties associated with conventional media design.

Statistical techniques such as Plackett-Burman design [25] are of particular help in eliminating the problems associated with conventional media optimization, which deals with a large number of experiments. This design is widely used by several researchers for reliable short-listing of a few parameters for further optimization. In microbial fermentation technology, statistical methods have been used for media optimization for production of a number of products, such as alcohols [26], polysaccharides [27] and enzymes [28]. Once the factors influencing the bioprocess are identified, it is necessary to determine their optimum levels. Conventional method of single factor optimization does not depict the combined effects of all the factors involved. These limitations of the conventional approach can be eliminated by optimizing all the affecting parameters collectively by statistical experimental design [29,30] using response surface methodology [31]. Various researchers have applied the technique for optimization of culture conditions, and process parameters such as pH, temperature, aeration, and feed rate [32].

Although statistical media design has been widely used in microbial fermentations for optimization of both growth and product formation, it has rarely been used in plant cell cultures. Two different statistical experimental designs have been employed for media optimization in plant cell cultures, *viz.*, the orthogonal design method and the response surface method. Orthogonal design method has been used for the optimization of growth and secondary metabolite production by cell suspension of *Cassia didymobotrya* [33]. Response surface method has been adopted for optimization of growth and secondary metabolite production by *Digitalis lanata* [34], and for optimization of indole alkaloid production by cell suspension cultures of *Catharanthus roseus* [35]. Plackett-Burman and response surface methodology have been used to optimize the culture and environmental parameters (glucose, indole-3-acetic acid, inoculum and initial pH) for cell growth and podophyllotoxin production by *Podophyllum hexandrum* suspension cultures; this enhanced the production of podophyllotoxin from 4.26 mg/L to 13.8 mg/L in stirred tank bioreactor [36].

PLANT CELL BIOREACTORS

Mass cultivation of plant cells *in vitro* is a viable alternative for production of high value, low volume phytochemicals. Considerable attention has, therefore, been given to design of bioreactors for large scale plant cell cultivation during the last two decades [6,7,9,10,12]. The problems of clumping of cells, mixing, oxygen transfer, shear sensitivity and wall growth necessitate a more rigorous analysis of bioreactors to be used for the cultivation of plant cells. Since the oxygen requirement, sensitivity to shear, rheological characteristics and the size of the cell aggregates vary from one plant species to the other, the selection of bioreactor depends largely upon the plant cell of interest. A suitable bioreactor can be designed for a specific plant cell system from the following considerations [6, 9, 12]:

- optimum aeration-agitation condition with respect to capacity of oxygen supply and intensity of hydrodynamic stress effects on the plant cells.
- intensity of culture broth mixing and air-bubble dispersion.
- control of temperature, pH and nutrient concentration inside the bioreactor.
- control of aggregate size (which may be important to enhance secondary metabolite production).
- maintenance of aseptic conditions for relatively longer cultivation period.
- ease of scale up.

A number of different types of bioreactors (Fig. 1) have been used for mass cultivation of plant cells taking the above considerations into account. Stirred tank bioreactors have been the most extensively applied in order to achieve the optimum process parameters. In spite of the fact that stirred tank reactors exert more hydrodynamic stress on plant cells, they have great potential when used with low agitation speed and modified impeller. The first commercial application of large scale cultivation of plant cells was carried out in stirred tank reactors of 200 L and 750 L capacities to produce shikonin by cell cultures of *Lithospermum erythrorhizon* [4]. Cells of *Catharanthus roseus* [37], *Dioscorea deltoidea* [19], *Digitalis lanata* [38], *Panax notoginseng* [39], *Taxus baccata* [40] and *Podophyllum hexandrum* [15] have been cultured in stirred tank bioreactors with suitable modifications for product formation. Another type of reactor known as bubble column reactor has also been used for large scale cultivation of plant cells. The major advantages of this reactor are the absence of moving parts and ease of maintaining sterile environment, as no sealing parts are required. *Cudrania tricuspidata*, being highly shear sensitive, was cultivated in bubble column reactor [12]. A modification of bubble column reactor 'balloon type bubble bioreactor' has been recently adopted for the production of taxol by *Taxus cuspidata* [41]. The major disadvantage of this reactor is insufficient mixing. A reactor having more uniform flow pattern with slight modification of stirred tank reactor (a draught tube is inserted instead of the impeller) is air-lift bioreactor. The cells of *Catharanthus roseus* [42], *Digitalis lanata* [43], *Cudrania tricuspidata* [12,44], *Lithospermum erythrorhizon* [44], and *Taxus chinensis* [45] have been success-

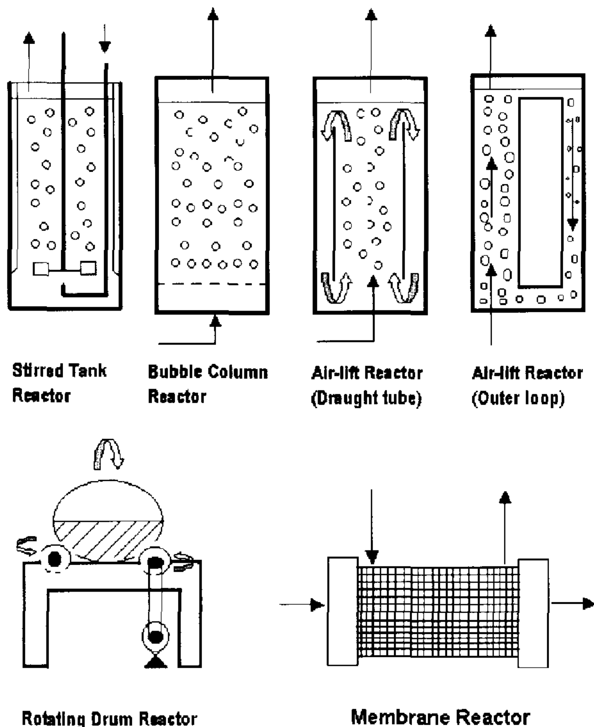

Fig. 1. Configurations of different bioreactors used for plant cell cultivation

fully cultivated in air-lift bioreactors for production of secondary metabolites. The major disadvantages of this reactor are the development of dead zones inside the bioreactor, insufficient mixing at high cell densities and rupture of cells due to collision between air bubbles and the cells. Another type of reactor used in plant cell cultivation is rotating drum reactor, which has higher oxygen transfer ability and relatively lower hydrodynamic stress. This consists of a horizontally rotating drum on rollers connected to a motor. Rotary drum reactor has been shown to be superior over other reactors for the cultivation of *Vinca rosea* [46] and *Lithospermum erythrorhizon* [44]. The performance of the different bioreactors has been summarized by Panda and co-workers [6].

PROCESS STRATEGIES

Selection of Cultivation Techniques

Various modes for culturing plant cells have been employed in suspension culture in order to maximize product formation. The fed-batch mode is used in cases where the addition of a high concentration of substrate affects the growth. The technique of repeated batch cultivation (semi-continuous mode) provides an appropriate approach towards the continuous cultivation of plant cells when the rate of product synthesis (or biotransformation of added precursors) parallels the rate of growth. For non-growth-associated products, the use of a two-stage culture, where cells are propagated in a growth medium and then transferred to a production medium, would be the ideal choice for maximizing product synthesis. Obviously, it would be important to recognize the best physiological state of the cell for maximal product accumulation. Once the type of bioreactor is selected for a specific plant cell process, the mode of operation will depend on the dynamics of the specific culture [47].

Batch cultivations are characterized by constantly changing environmental conditions, and are capable of producing metabolites associated with any kinetic pattern. Therefore, a number of plant cell systems have been cultivated under batch mode to scale-up the process. Although batch cultivation strategy has been widely adopted for scale-up of plant cell bioprocesses, it has not always been successful in improving the production of desired metabolites; in many cases the production of secondary metabolites has been reported to be decreased in scale-up process. Batch studies are convenient systems for determination of the suitable conditions for maximum productivity. A variety of plant cells cultivated under batch mode for production of secondary metabolites are summarized in Table 4. Stirred-tank bioreactors with modified impellers that impart improved mixing under low shear have been advocated for a long time for cultivation of fragile plant cells in large scale suspension cultures. Although most studies of this kind have addressed the effect of mechanical agitation on cell growth, the direct effect of various agitation conditions on cell growth and metabolism in a batch reactor has also been investigated [48]. The effect on power consumption and flow pattern was also investigated. The type of impeller, and the agitation speed, all of which affect the rate of shear inside the reactor, have been studied in the suspension culture of *Nicotiana tabacum*. A large flat-bladed turbine impeller with a high width-to-diameter ratio imparted more gradual velocity gradients than the regular flat-bladed disc turbine, thus producing well distributed mixing patterns without dead zones at low shear. All of these factors contributed towards more efficient growth and enhanced metabolites (phenolics) production. Evidence that high shear contributed to more cell damage was provided by the use of a large flat-bladed impeller with sail cloth blades, that resulted in higher maximum growth because of the higher viability of the cells. In the same study [48], it was also observed that the cells were more susceptible to shear damage during the decelerating log phase of growth. Thus, the use of different agitation speeds during different stages of growth would be a more rational approach to the successful cultivation of plant cells in suspension. *Panax ginseng* has been successfully cultivated at a large scale in 2,000 L and 20,000 L stirred tank bioreactors to produce 500–700 mg L^{-1} day^{-1} of ginseng saponins [49]. *Panax ginseng* cell lines have been cultivated in both stirred tank and air-lift bioreactors for the production of ginsenoside. Different types of impellers (flat-blade, angled-blade disc turbine, anchor impeller) at various impeller speeds have been used for

the cell growth of *Panax ginseng* and it has been observed that angled-blade disc impeller at 100-150 rpm resulted in highest cell growth, indicating the shear sensitivity of the cells [50]. It was also observed that the impeller speed was the most significant parameter affecting the production of ginsenosides in the bioreactors. With another cell line of *P. ginseng* cultivated in a 2 L stirred tank bioreactor with a marine propeller, the cells could grow fairly well up to an unusually high agitation speed of 1,000 rpm [50], indicating shear-resistant nature of the cell line. In another study, the shear stress exerted by aeration and agitation on cultured *Coptis japonica* cells in stirred tank bioreactor was minimized by the use of oxygen-enriched air with a hollow-paddle type agitator [51,52].

The conditions derived from the batch cultivation form the basis of substrate and/or product inhibition and, therefore, can be used to design suitable fed-batch or continuous operation to overcome the inhibitions by controlled addition of a limiting nutrient. Fed-batch cultivation has been able to improve the productivity of ginseng by *Panax ginseng* [53], and taxane by *Taxus chinensis* [54]. Fed-batch cultivation of *Coptis japonica* had a significant effect on production of berberine at high cell density, as batch cultivation in stirred tank bioreactor damaged the cells due to high osmotic pressure of the culture medium. Further, the biomass concentration was reduced due to accumulation of inhibitory products during cell growth. This problem was resolved by suitable fed-batch cultivation, which enhanced both cell growth and berberine production [51, 52]. An increased production of cinnamoyl putrescines has also been observed by fed-batch cultivation of *Nicotiana tabacum* in stirred tank bioreactor [55]. Production of podophyllotoxin has been enhanced to 43.2 mg/L by fed-batch cultivation of *Podophyllum hexandrum* in stirred tank bioreactors as compared to 13.8 mg/L in batch cultivation (authors' work) (Table 3).

Steady state continuous flow or chemostat operation, with a constant withdrawal of culture medium and cells is commonly used for the production of growth-associated products, typically primary metabolites and biomass. It also provides a system to eliminate product inhibition, if any. The continuous culture technique has also been adopted for the cultivation of several plant cells such as, *Coptis japonica* [51], *Catharanthus roseus* [67], and *Nicotiana tabacum* [68]. A high cell density of *Coptis japonica* produced 3500 mg/L berberine when cultivated in continuous mode in 2.5 L stirred tank bioreactor [51,52]. However, the cellular content of berber-

Table 3. Production of secondary metabolites by plant cell suspension cultures under different modes of cultivation

Plant cell	Product	Bioreactor type, capacity and mode of cultivation	Product (mg/L)	Reference
Anchusa officinalis	Rosmarinic acid	Stirred tank bioreactor, 2.5 L, batch	3,500	[56]
Aralia cordata	Anthocyanin	Jar culture vessel, 500 L, continuous	1,090	[57]
Catharanthus roseus	Ajmalicine	Air-lift bioreactor, 20 L, batch	6.4	[58]
	Catharanthine		3	[58]
	Serpentine		1.6	[58]
	Tryptamine		16.1	[58]
Coptis japonica	Berberine	Stirred tank bioreactor, 2.5 L Batch	800	[51]
		Fed-batch	2,320	[51]
		Continuous	3,500	[51]
Holarrhena antidysenterica	Conessine	Stirred tank bioreactor, 6 L, batch	106	[59]
Lithospermum erythrorhizon	Shikonin	Stirred tank bioreactor, 200 and 750 L, two-stage culture	4,000	[60]
Nicotiana tabacum	Cinnamoyl putrescines	Stirred tank bioreactor		
		Batch	160	[55]
		Fed-batch	400	[55]
Panax notoginseng	Ginseng saponin	Centrifugal impeller bioreactor, 2.5 L, batch	800	[61]
		Turbine bioreactor, 2.5 L, batch	490	[61]
		Air-lift bioreactor, 1 L, batch	3,120	[62]
		Erlenmeyer flask, 0.25 L, batch	1,570	[63]
Perilla frutescens	Anthocyanin	Erlenmeyer flask, 0.5 L	5,800	[64]
Podophyllum hexandrum	Podophyllotoxin	Stirred tank bioreactor, 3 L batch	13.8	[36]
		Fed-batch (intermittent feeding)	43.2	Authors' work
		Continuous with cell retention	48.8	Authors' work
Taxus chinensis	Taxane	Erlenmeyer flask, 0.25 L	274.4	[54]
	Paclitaxel	Erlenmeyer flask, 0.25 L Semi-continuous batch	85.5	[65]
Taxus cuspidata	Taxol	Wilson type bioreactor	22	[66]

ine in continuous culture decreased to less than 50% of that observed in batch cultivation because the production of berberine in *C. japonica* was a part of the non-growth-associated kinetics (Table 3).

Cell retention systems have been occasionally employed for the enhancement of growth and product yield in various microbial systems for their ability to achieve high cell density in continuous cultivation. *In situ* cell retention systems have been particularly successful in improving the productivity of product inhibited cultivations, mainly because the bioreactor could be operated at high dilution rates to flush out inhibitory products and at the same time the cells could be retained by the filtration device [69]. Spin filter device has been applied for the somatic embryogenesis of plant cell suspension cultures and for industrial plant propagation [70]. *Podophyllum hexandrum* has been cultivated in stirred tank bioreactor in continuous mode using cell retention device and this further enhanced the production of podophyllotoxin to 48.8 mg/L (authors' work) (Table 3).

A study on growth of *Catharanthus roseus* suspension cultures for alkaloid production was carried out in shake flask and air-lift bioreactors (7 L and 30 L) using batch, fed-batch and draw-fill modes, and it was observed that scale-up led to reduction in serpentine production. Although cell growth could be enhanced by fed-batch cultivation, the alkaloid accumulation was retarded [71]. Because of the non-growth-associated kinetics of secondary metabolite production, a two-stage process would be more suitable for enhanced product synthesis. A two-stage process has been employed successfully for the production of deacetyl lantoside C by *Digitalis lanata*. Using a second bioreactor containing glucose only, 85% conversion efficiency of β-methyl digitoxin could be achieved [72]. However, the application of two-stage culture strategy to berberine production by *Thalictrum rugosum* did not yield favorable results [73], apparently because berberine synthesis in *T. rugosum* was not a non-growth-associated event.

The application of semi-continuous cultivation mode of *D. lanata* cell lines in a 300-L air-lift bioreactor has also been carried out successfully for biotransformation of digitoxin to digoxin. Repeated batch cultivation using the same bioreactors, employing six consecutive runs, greatly enhanced the productivity of the system [43]. Therefore, the recognition of the most favorable physiological state of secondary metabolite synthesis is the prime factor that guides the type of cultivation mode for the optimum productivity in large scale bioreactors.

Use of Mathematical Models

Mathematical models are advantageous for understanding the effects of various process parameters controlling a specific system. The design of bioreactors and selection of cultivation strategy is guided by suitable mathematical models. They help understand the system and guide further experimental efforts. However, only a few cases exist where mathematical modeling has been used for improvement of plant cell processes. Structured, non-segregated models, which assume biomass distribution between two or more compartments, have been used to describe cell growth and secondary metabolite production in plant cell cultures [74,75]. A mathematical model has recently been applied for improving the production of polysaccharides by *Symphytum officinale* suspension cultures [76]. Mathematical modeling has also been found to be useful for embryogenesis of *Betula pendula* [77] and optimization of the production of capsaicinoid by immobilized *Capsicum frutescens* cells [78].

In addition to oxygen, which plays a major role in the energetics of plant cells and probably also in plant differentiation, carbon dioxide has been reported to improve cell growth and metabolite synthesis in a few plant cell cultures. Thus, the level of these gases needs to be controlled for their optimal utilization in a bioreactor. Using a mathematical model which accounts for gas-liquid mass transfer, biological utilization and production of oxygen and carbon dioxide, and the series of chemical reactions of carbon dioxide in water, it has been possible to control the dissolved oxygen and carbon dioxide concentrations by manipulating the gas composition at the inlet to the bioreactor. The control system, which employed the mathematical model in an adaptive feed-forward strategy, allowed the dissolved concentrations of the gases to be controlled without changing either the total gas flow rate or the agitation speed, thereby maintaining constant shear conditions in the bioreactor. The control strategy has been shown to be applicable for cultivation of *C. roseus* cells [79].

Precursor Feeding

Precursors of biosynthetic pathways have been used in various plant cell suspension cultures to improve the production of secondary metabolites. Factors such as the concentration and the time of addition of the precursor are to be considered when applying the precursor to the cell culture medium. The addition of loganin, tryptophan and tryptamine enhanced the production of secologanin [80], and indole alkaloids [81] by *Catharanthus roseus* suspension cultures. Paclitaxel yields in the cell culture of *Taxus cuspidata* were improved up to six times by feeding phenylalanine and other potential paclitaxel side-chain precursors (*e.g.* benzoic acid, *N*-benzoylglycine and serine) [82]. Cholesterol, a precursor of alkaloid biosynthesis, was found to have a strong effect on the production of conessine by *Holarrhena antidysenterica* cell suspension culture [59]. The time of addition of cholesterol as well as its concentration had a significant effect on alkaloid synthesis. A step feeding strategy, in which 50 mg/L cholesterol was added in 4 instalments during different phases of growth, enhanced the production of the alkaloid from 63 mg/L to 106 mg/L in 6-L stirred tank bioreactor; this study highlighted the importance of the physiological state of the culture for effective transformation of the precursor to alkaloid [59] (Fig. 2).

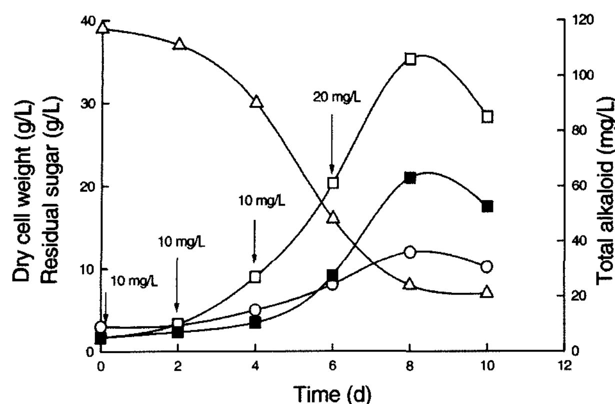

Fig. 2. Growth and alkaloid production by *H. antidysenterica* cell cultures with step feeding of cholesterol in 6 L stirred tank bioreactor [○ dry cell weight, □ total alkaloid (with cholesterol), ■ total alkaloid (without cholesterol), and △ residual sugar] [59].

Permeabilization of Plant Cells

Plant secondary metabolites are normally produced intracellularly which adds up to the cost of downstream processing of a specific product. It is, therefore, desirable to extract the products into the culture medium such that the purification procedure becomes easier. Removal of secondary metabolites from the vacuoles of the cells would also reduce the product inhibition and increase the productivity. Many attempts have been made to permeabilize the plant cell membranes in a reversible manner with organic solvents. Dimethylsulfoxide (DMSO) has been used in many cases, because it is known to extract sterols from the membranes of the eukaryotic cells. Of various cells tested, only *Catharanthus roseus* survived the treatment of DMSO [83]. Various other organic solvents such as hexadecane, perfluorchemicals and Miglyol have been employed for the extraction of the intracellular plant products without affecting the cell viability. Taxol has recently been extracted by various organic solvents such as hexadecane, decanol and dibutylphthalate, in the range of 5-20% (v/v), in the culture medium of *Taxus chinensis* [84]. Selection of a specific solvent system with due consideration to its effect on cell growth may lead to substantial release and increase in the production of secondary metabolites.

Some general rules of thumb have been reported for the prediction of biocatalytic activity in the presence of a solvent [85]. The value of 'log P' (P is the partition coefficient of the solvent over a standard octanol/water two-phase system) of a solvent was used to predict its influence on the retention of biocatalytic activity. On the basis of several experiments with enzymes and microorganisms, it has been hypothesized that when the 'log P > 4' (*e.g.* undecanol, hexadecane, and dioctylthalate), there was no negative influence, when 'log P < 2' (*e.g.* ethylacetate), biocatalytic activity of the solvent decreased drastically, and when '2 < log P < 4' (*e.g.* diethylphthalate and hexane), the activity of the solvent varied. The hypothesis has been experimentally tested with suspension cultures of *Tagetes minuta* [86] and with hairy root cultures of *T. patula* [87] and it has been observed that the solvents with 'log P > 5' (*e.g.* decane, and hexadecane) had no adverse effect on growth and product formation.

FUTURE PROSPECTS

Plant cell cultivation is a suitable alternative to whole plant cultivation for the production of desired compounds. However, due attention must be given to the biological and engineering parameters related to the growth of and secondary metabolite production by plant cells in suspension cultures. The inherent difficulties associated with *in vitro* plant cell cultivation e.g. genetic variation of plant cell lines, sensitivity to shear stress, complex regulatory mechanism etc. are to be properly addressed for a specific cell line. Design of a suitable bioreactor with low-shear impeller, and selection of an appropriate mode of cultivation is required for increased metabolite production. Optimization of medium ingredients by statistical techniques, application of appropriate mathematical models for optimized cell cultivation, feeding strategy of metabolic precursors and extraction of intracellular metabolites by organic solvents can lead to significant enhancement in productivity of secondary metabolites.

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